

Plasma Concentrations and Pharmacokinetic Parameters of Nitrofenac Using a Simple and Sensitive HPLC Method

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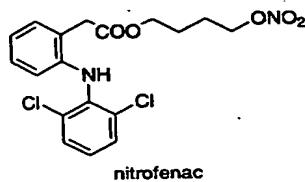
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Abstract □ An accurate and sensitive HPLC method has been developed for the determination of nitrofenac, a new, original diclofenac derivative showing a good tolerability and a wide anti-inflammatory profile, diclofenac, and its metabolites in plasma. This method has been applied to evaluate the pharmacokinetic parameters of the drugs, using a noncompartmental model, after the oral administration of 5 mg/kg nitrofenac to rats. Nitrofenac and the internal standard flufenamic acid were dissolved in acetonitrile, and diclofenac was dissolved in methanol. The drugs were eluted from a 5 μm LC-8 column with a mobile phase consisting of acetonitrile/water (50/50 v/v) adjusted to pH 3.3 with glacial acetic acid, at a flow rate of 2 mL/min with UV detection at 280 nm for diclofenac and 275 nm for nitrofenac. The detection limit for the drugs in plasma was 25 ng/mL. The peak concentration of nitrofenac was reached 7 h after drug administration, while with diclofenac we observed three peaks at 2, 5, and 10 h; the mean residence time and the elimination rate constant for nitrofenac were 6.18 ± 0.09 h and $0.37 \pm 0.03 \text{ h}^{-1}$ respectively, while those for diclofenac were 12.24 ± 0.11 h and $0.11 \pm 0.04 \text{ h}^{-1}$. Under our conditions, the metabolism of nitrofenac produced 23% diclofenac and other metabolites: the plasma concentrations and kinetic characteristics of diclofenac are enough to induce an anti-inflammatory activity, while the clinical importance of the other metabolites remains to be elucidated.

Introduction

The sodium salt of diclofenac is a potent anti-inflammatory and analgesic drug, widely employed in rheumatology.^{1,2}



Nitrofenac, 2-[2,6-dichlorophenyl]amino]benzeneacetic acid 4-(nitrooxy)butyl ester, is a new, original diclofenac derivative showing a better tolerability and a wider anti-inflammatory profile than diclofenac, obtained by a molecular manipulation consisting of the insertion of a nitric oxide side chain. It is a drug soluble in dimethyl sulfoxide, methylene chloride, and methanol and insoluble in water, with a MW = 412. It is stable in the solid state at room temperature, if protected from light.

A variety of analytical techniques are available for the quantitation of diclofenac sodium in biological fluids, including thin layer chromatography,³ gas-liquid chromatography,^{4,5} and high-performance liquid chromatography.^{6,7} In particular, several HPLC techniques have been reported for deter-

mination of diclofenac sodium in plasma, urine, and synovial fluid, but these more complex, time-consuming, and tedious extraction steps⁷⁻⁸ are not suitable for kinetic studies.⁹

This paper describes a simple, accurate, sensitive, and reproducible HPLC assay for the determination of nitrofenac, diclofenac and its metabolites in plasma.

Experimental Section

Protocol—Ninety-six male Sprague-Dawley rats from Charles River S.p.A. (Calco, Italy), weighing 202 ± 30 g, were used. Nitrofenac, at the dose of 5 mg/kg, was suspended in 5% (carboxymethyl)-cellulose and administered orally in a volume of 1 mL/100 g of body weight.

Blood samples, taken by intracardiac puncture, were collected in EDTA-containing tubes at 30 min and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 h after drug administration, centrifuged at 3000 rpm for 10 min, analyzed immediately, and stored at -20°C .

Apparatus—The HPLC equipment used comprised a 420 pump, a variable wavelength detector (model 430), and an autosampler 460 (Kontron Instr, Zurich, Switzerland). The detector signal was processed by a PC 450 data system, from Kontron Instr.

Chromatographic separations were performed using Supelcosil 5 μm LC-8 column (4.6 \times 250 mm, Supelco Inc., Bellefonte, PA).

Reagents—All solvents used were HPLC grade; all other chemicals and reagents were of spectroquality or analytical grade. Diclofenac sodium and flufenamic acid were obtained from Sigma Chemical Co. (St. Louis, MO), and nitrofenac was from Metgrove Ltd (London, U.K.). A patent for nitrofenac was applied for in Italy and will be extended world wide in August 1993.

The mobile phase comprised acetonitrile/water (50/50 v/v) adjusted to pH 3.3 with glacial acetic acid. The mobile phase was degassed daily by passing it through a 0.45 μm membrane filter (Millipore, Bedford, MA).

Standard Solutions—Diclofenac sodium was dissolved in methanol (1 mg/mL). Flufenamic acid and nitrofenac were dissolved in acetonitrile (1 mg/mL). The stock solutions were diluted 10- and 100-fold in methanol and acetonitrile, respectively, to give the working standard solutions.

Extraction Procedure and Analysis—In a 10 mL culture tube, diclofenac sodium and nitrofenac working standards (10 $\mu\text{g/mL}$ = 1:100 or 100 $\mu\text{g/mL}$ = 1:10) were added to 1 mL of plasma to provide calibration standards. Diclofenac sodium was added in volumes of 0, 5 (1:100), 10 (1:100), 20 (1:100), 100 (1:100), 200 (1:100), and 50 (1:10) μl to provide calibration standards of 0 (no diclofenac sodium standard added), 50, 100, 200, 1000, 2000, and 5000 ng/mL (range, 50–5000 ng/mL). Nitrofenac was added in volumes of 0, 20 (1:100), 100 (1:100), 200 (1:100), 50 (1:10) μl to provide calibration standards of 0 (no nitrofenac standard added), 200, 1000, 2000, 5000 ng/mL (range, 200–5000 ng/mL). Calibration standards and plasma samples followed the same extraction procedure. An appropriate volume (1 mL) was poured into a glass-stoppered tube with the appropriate amount of internal standard solution (500 ng of flufenamic acid). Precipitation of serum proteins was accomplished by addition of 4 mL of acetonitrile. The mixture was shaken on a vortex mixer for 1 min and centrifuged for 10 min at 2500 rpm. The supernatant, transferred to a 10 mL centrifuge tube, was evaporated to dryness at 45°C in a water bath under a stream of dry nitrogen. The residue was reconstituted in 200 μl of HPLC eluent, vortexed for 30 s, transferred to a disposable polypropylene microcentrifuge tube (1.5 mL, Eppendorf), and centrifuged for 2 min at 11 500 rpm to ensure

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that no particulate matter would be injected into the column. An appropriate aliquot (140 μ L) was then injected directly into the loop injector.

The mobile phase was pumped at a flow rate of 2 mL/min for the first 13 min, gradually set to 2.7 mL/min in 17 min, held for 11 min, and finally taken to the initial conditions: the resulting analysis time was 44 min. The chart speed was 2.5 mm/min; the effluents diclofenac and flufenamic acid were monitored at 280 nm, while nitrofenac at 275 nm.

The quantitation of the chromatogram was performed using peak area ratios of the drugs to the internal standard: a representative standard curve of diclofenac and nitrofenac–flufenamic acid peak area ratio resulted in the following linear least-squares regression equations respectively: $y = 0.000 + 0.213x$ ($r = 1$) and $y = -0.001 + 0.522x$ ($r > 0.999$).

Standard curves of diclofenac sodium and nitrofenac were constructed on four different days to determine the coefficient of variability of the slope: 7.1% and 11.1% for diclofenac sodium and nitrofenac, respectively. The intraday precision (random analytical variation) was evaluated by replicate analysis of plasma samples containing diclofenac sodium and nitrofenac at a known concentration. The intraday precision showed a coefficient of variation of 1.1% for diclofenac sodium (accuracy = 0.3%) and of 6.2% for nitrofenac (accuracy = 3.2%). The interday precision (total analytical variation) was evaluated in a 2 week period of time. The interday CV_s was 2.7% for diclofenac sodium (accuracy = 2.4%) and 7.4% for nitrofenac (accuracy = 6%).

The limit of detection for this method was 25 ng/mL for diclofenac sodium and 100 ng/mL for nitrofenac (the limit of quantitation was obtained by diluting scalarly the last point of the calibration curve constructed with plasma samples).

Pharmacokinetic Parameters—The pharmacokinetic model chosen to describe the course of the plasma concentrations was noncompartmental: the parameters were calculated according to the formulas of Gibaldi and Perrier.¹⁰

Statistical Analysis—For the comparison of the data, the Student's "t" test was applied.

Results

Diclofenac and flufenamic acid were monitored at 280 nm as described previously, while nitrofenac was monitored at 275 nm. To better evaluate the absorption of nitrofenac, we monitored different drug concentrations at four different λ values, using a UV "diode array" detector: the procedures, using a Kontron system, confirmed 275 nm to be the best for nitrofenac determination.

Moreover, we evaluated the nitrofenac concentrations in water and plasma, and no differences were revealed, showing that the plasma drug extraction was complete and excellent. No interferences from other components in plasma were observed.

In Figure 1 are shown the chromatograms referred to as the blank plasma extract, the plasma sample spiked with the drugs and the internal standard, and the plasma sample from the rat study.

Figure 2 and Table 1 show the plasma concentration–time profile of nitrofenac and its metabolite diclofenac after administration of 5 mg/kg nitrofenac. The peak concentration of nitrofenac was reached after 7 h from drug administration ($C_{max} = 1.36 \pm 0.31 \mu\text{g}/\text{mL}$) and at 24 h nitrofenac was not detectable in the plasma with our techniques. With diclofenac, we observed three peaks at 2, 5, and 10 h (0.211 ± 0.02 , 0.324 ± 0.19 , and $0.120 \pm 0.01 \mu\text{g}/\text{mL}$, respectively).

In Table 2 are shown the pharmacokinetic parameters of the two drugs: the mean residence time and the elimination rate constant for nitrofenac were $6.18 \pm 0.09 \text{ h}$ and $0.37 \pm 0.03 \text{ h}^{-1}$, respectively, while for diclofenac they were $12.24 \pm 0.11 \text{ h}$ and $0.11 \pm 0.04 \text{ h}^{-1}$.

The disappearance rate of nitrofenac was much more rapid than that of diclofenac (Figure 2 and Table 2), which is highly bound (>99.5%) to serum proteins, mostly to albumin. Mor-

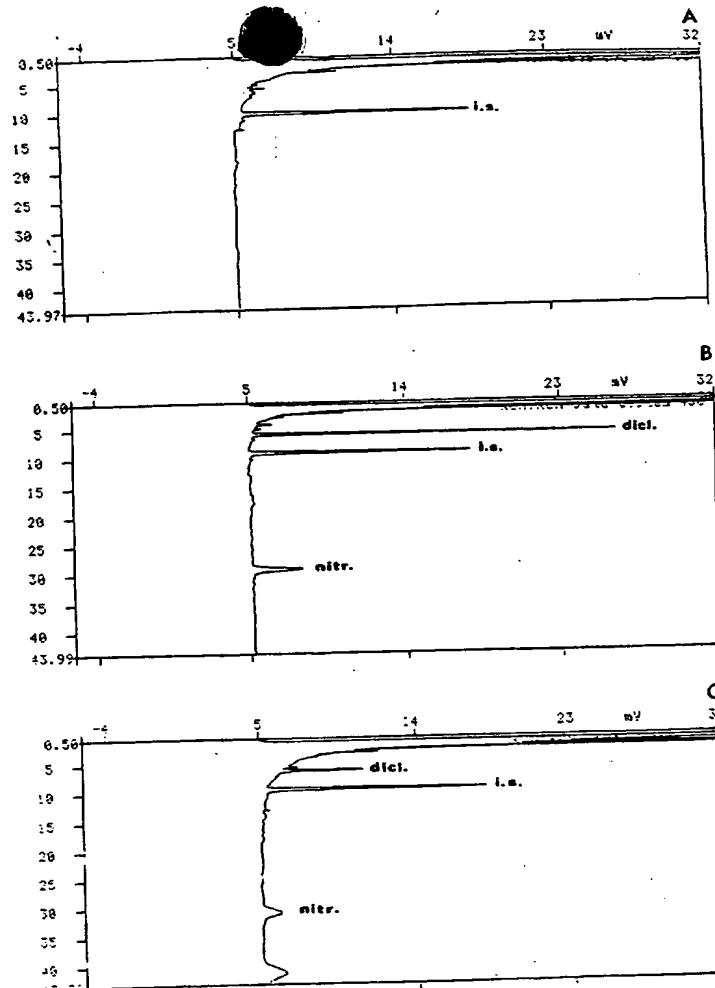


Figure 1—Chromatograms showing (a) blank plasma extract with internal standard (flufenamic acid); (b) plasma sample spiked with diclofenac (1 $\mu\text{g}/\text{mL}$), internal standard, and nitrofenac (1 $\mu\text{g}/\text{mL}$); (c) plasma sample from the rat study.

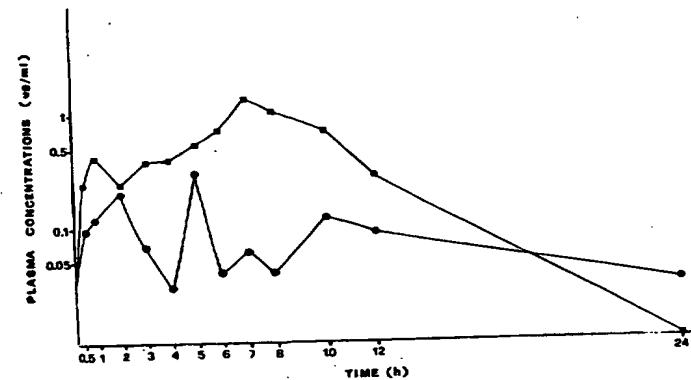


Figure 2—Nitrofenac (■) and diclofenac (○) plasma concentrations after oral administration of 5 mg/kg nitrofenac.

over diclofenac penetrates tissues, particularly heart and lung, and this takes into account the large volume of distribution at steady state in addition to the high protein binding.

The ratio between the $AUC_{0-\infty}$ of diclofenac and nitrofenac shows that 23% of the parent drug is metabolized. The dose of diclofenac was calculated as a percentage of the nitrofenac dose. Moreover, in the first 7 h it has been identified as a

Table 1—Nitrofenac and Diclofenac Plasma Concentrations in Rats Treated Orally with 5 mg/kg Nitrofenac^a

Time (h)	Nitrofenac ($\mu\text{g/mL}$)	Diclofenac ($\mu\text{g/mL}$)	Time (h)	Nitrofenac ($\mu\text{g/mL}$)	Diclofenac ($\mu\text{g/mL}$)
0.5	0.25 \pm 0.01	0.096 \pm 0.01	6	0.71 \pm 0.20	0.040 \pm 0.01
1	0.44 \pm 0.17	0.113 \pm 0.05	7	1.36 \pm 0.31	0.062 \pm 0.02
2	0.25 \pm 0.10	0.211 \pm 0.02	8	1.02 \pm 0.35	0.040 \pm 0.01
3	0.39 \pm 0.01	0.068 \pm 0.01	10	0.72 \pm 0.14	0.120 \pm 0.01
4	0.40 \pm 0.01	0.030 \pm 0.01	12	0.29 \pm 0.15	0.090 \pm 0.02
5	0.56 \pm 0.19	0.324 \pm 0.19	24	0	0.032 \pm 0.29

Table 2—Nitrofenac and Diclofenac Pharmacokinetic Parameters Using a Noncompartmental Model^b

	Nitrofenac	Diclofenac
C_{\max} ($\mu\text{g/mL}$)	1.36 \pm 0.31	0.32 \pm 0.19
T_{\max} (h)	7.00 \pm 0.02	5.00 \pm 0.01
$AUC_{0-\infty}$ ($\mu\text{g}/\text{mL}\cdot\text{h}$)	10.01 \pm 0.75	2.28 \pm 0.05
AUC_{0-24} ($\mu\text{g}/\text{mL}\cdot\text{h}$)	9.22 \pm 0.25	1.99 \pm 0.07
β (h^{-1})	0.37 \pm 0.03	0.11 \pm 0.04
$AUMC_{0-\infty}$ ($\mu\text{g}\cdot\text{h}^2/\text{mL}$)	61.84 \pm 2.15	27.91 \pm 1.80
MRT (h)	6.18 \pm 0.09	12.24 \pm 0.11
% metabolite	$\frac{AUC_{0-\infty}(\text{diclo})}{AUC_{0-\infty}(\text{nitro})}$	23.0 \pm 0.15

^aThe values are expressed as mean \pm SE. C_{\max} = maximum plasma concentration; T_{\max} = time required for the maximum plasma concentration to be reached; AUC = area under the curve; β = elimination rate constant; C_t = total clearance; V_{dss} = volume of distribution at steady state; AUMC = area under the first moment curve; MRT = mean residence time.

metabolite with a retention time of about 40 min (Figure 1c), while in the following sampling times there have been identified other metabolites with retention times close to that of diclofenac (Figure 3).

Discussion

Nitrofenac is a new nitrate ester of diclofenac: it shows a chemical group CONO₂ with a nitrogen linked to the carbon atom via an oxygen atom.

Studies on the metabolism of nitrate esters developed originally from the efforts of earlier investigators to establish a relationship between their breakdown in vivo and the physiological effects produced. Litchfield,¹¹ in a review article, referred to reduction of the nitrate esters to the corresponding nitrite esters as the first step in metabolism, followed by hydrolysis to release inorganic nitrite.

Under our conditions, during the first 24 h after a single oral dose of 5 mg/kg to rats, the metabolism of nitrofenac produces 23% diclofenac, a known antiinflammatory drug. Moreover, beside the diclofenac, we detected other metabolites, presumably belonging to the alcoholic metabolite, devoid of the NO moiety. In every case, there were plans to study the release of nitric oxide after the administration of nitrofenac by measuring the plasma nitrate/nitrite levels at the Prof. Wallace Institute.

Plasma diclofenac concentrations, produced by nitrofenac metabolism, are enough to induce the antiinflammatory action: this observation was strengthened by another work in this field, where the oral administration of 5 mg/kg nitrofenac to rats produced a sharp reduction of carrageenan paw edema 3 and 5 h after drug administration.¹²

Diclofenac shows a first peak in the plasma followed, after the initial fall, by a secondary increase in plasma levels associated with a slow increase in nitrofenac concentrations: this could probably be explained by the intestinal degradation

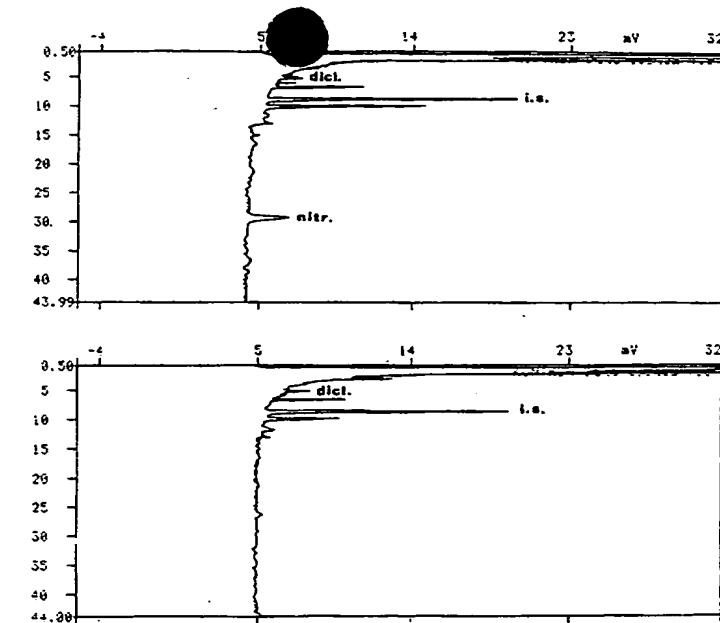


Figure 3—Chromatograms showing the metabolites with retention times close to that of diclofenac.

at first and then by the active plasma metabolism of the parent drug.

In addition, the significant plasma metabolism of nitrofenac takes into account the absence of drug at the 24 h sample collection. The rate of disappearance of nitrofenac is much more rapid than that of diclofenac: this drug persists for a long time in the body, as showed by the long mean residence time; moreover it is known that diclofenac shows high protein binding.¹³

In conclusion, nitrofenac is partially metabolized to diclofenac: the plasma concentrations and kinetic characteristics of this metabolite are enough to induce an antiinflammatory activity, as previously shown.¹² The clinical importance of the other detectable metabolites remains to be elucidated.

References and Notes

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